Probing the nucleotide binding sites in T7 RNA polymerase using cibacron blue

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T7 RNA polymerase (T7 RNAP) is an enzyme that utilizes ribonucleotides to synthesize the nascent RNA chain in a template-dependent manner. In this work we have studied the interaction of T7 RNAP with cibacron blue, an anthraquinone monochlorotriazine dye, and its effect on the function of the enzyme. The first phase of the binding is characterized by a high affinity ($K_d$ in the nanomolar range) and irreversible inactivation of the enzyme. The second binding site is the common substrate binding site. The association of the dye with T7 RNAP is a good model to understand the physiological significance of a high affinity binding of the initiating nucleotide, GTP, earlier reported from our laboratory. The results will be discussed to understand the role of the high affinity GTP binding.

Introduction

T7 RNA polymerase (T7 RNAP) is a single subunit enzyme of 99 kD molecular weight that can carry out all the processes of specific transcription including initiation, elongation and termination of the RNA chain without the help of any accessory factors in vitro and in vivo. T7 RNAP finds widespread use in biotechnology for expression of cloned and recombinant proteins\(^1\)\(^3\) and is extensively studied as a model system to understand polymerase-substrate interactions\(^4\)\(^6\) and mechanism of transcription\(^7\)\(^9\). The gene for T7 RNAP has been cloned and can be expressed in large amounts\(^11\).

Cibacron blue F3GA, a sulfonated, anthraquinone monochlorotriazine dye is widely used to probe specific domains in the structure of proteins by monitoring spectral and activity changes upon its binding to enzymes\(^12\). It has been suggested as a site-specific probe for nucleotide binding domains and dinucleotide folds in proteins because it was found to interact with many proteins or enzymes utilizing nucleotide substrates and nucleotide coenzymes\(^13\)\(^17\). DNA-dependent T7 RNA polymerase employs ribonucleotides as substrates and DNA as template to catalyze the synthesis of RNA chain and thus, it is expected to have cibacron blue binding sites.

In the present work, the interaction of T7 RNAP with cibacron blue F3GA and its subsequent effect on the transcriptional activity of the enzyme has been studied. The results show that cibacron blue binds reversibly to T7 RNAP in a bi-phasic manner. In the first phase, the dye binds with high affinity ($K_d$ in the nanomolar range) leading to a reversible inactivation of the enzyme. The high affinity association is followed by a binding with less affinity ($K_d$ in micromolar range) that is competitive with the nucleotide substrate binding site. Such a bi-phasic nature of association characterized by nanomolar order of dissociation constant has not been reported for any other RNA polymerase.

Materials and Methods

Materials

Tris, magnesium chloride, potassium chloride, spermidine, DTTF, EDTA RNAse A and cibacron blue were from Sigma Chemical Company (St. Louis, Missouri). Ultrapure nucleoside triphosphates and MonoQ HR 5/5 were obtained from Pharmacia Fine Chemicals, Sweden. [\(\alpha\)^32P\]UTP and [\(\alpha\)^32P\]GTP were purchased from BRIT, India. All other reagents used were of analytical grade.

Isolation and linearization of plasmid DNA

Escherichia coli strain XL1 blue was earlier transformed with the template plasmid DNA, pARC035, containing the strong T7 RNAP promoter, \(\phi H 10\). The plasmid was then isolated from it by the alkaline lysis method described by Sambrook\(^18\). It was purified from the contaminating RNA by treatment with RNAase A followed by extraction with phenol and chloroform according to standard procedures. The purity of the plasmid DNA was checked by agarose-
gel electrophoresis and this DNA was used as the template for in vitro transcription assays.

Isolation and purification of T7 RNA polymerase

T7 RNAP was isolated according to the method described by Grodberg and Dunn and purified by two modified methods developed in our laboratory. The purity of the enzyme preparation was checked by SDS-PAGE followed by silver staining. The concentration of T7 RNAP was determined from its absorbance at 280 nm using an e value of \(1.4 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}\).

Absorption measurements of cibacron blue bound to T7 RNA polymerase

Difference in absorption spectra of cibacron blue in absence and presence of T7 RNAP were recorded from 400 nm to 750 nm in a Hitachi U3300 spectrophotometer. Baseline difference spectrum was recorded first with TDMK buffer (50 mM Tris-HCl, pH 8.0, containing 50 mM KCl, 10 mM MgCl₂ and 1.24 mM DTT) in both sample and reference cuvettes. 1.5 \(\mu\)M T7 RNAP was then added to the sample cuvette and an equal volume of buffer was added to the reference cuvette to record the spectrum. Equal aliquots from a concentrated stock solution of cibacron blue were added to both cuvettes and the difference spectra were recorded after each incremental addition. Cibacron blue concentration in the cuvettes was varied from 68 nM to 5.0 \(\mu\)M. The stoichiometry of binding in terms of number of bound dye molecules per T7 RNAP molecule was determined from the ratio of the point of intersection of the two straight lines of the plot of difference absorption at 686 nm against dye concentration, and the enzyme concentration (1.5 \(\mu\)M).

Fluorescence measurements

Fluorescence spectra of 1.0 \(\mu\)M T7 RNAP in TDMK buffer, pH 8.0, in the absence and presence of different concentrations of cibacron blue were recorded in a Shimadzu FS40 spectrofluorometer. The enzyme was titrated with increasing concentrations of cibacron blue and the emission intensity at 340 nm \((\lambda_{ex} = 295 \text{ nm})\) as a function of input concentration of cibacron blue was recorded to get the titration profile. Inner filter corrections were done for absorbance values of the dye greater than 0.05 according to the equation:

\[
F_{\text{corr}} = F_{\text{obs}} \times \text{antilog} \left[ \left( A_{\text{ex}} + A_{\text{em}} \right) / 2 \right]
\]  \hspace{1cm} (1)

where, \(F_{\text{corr}}\) is the corrected emission intensity, \(F_{\text{obs}}\) is the observed emission intensity, \(A_{\text{ex}}\) and \(A_{\text{em}}\) are the absorbance of cibacron blue at the excitation and emission wavelengths, respectively. Dissociation constant \((K_d)\) was evaluated from the mid-point of transition at each phase of the binding profile.

Effect of cibacron blue binding on polymerase activity

For T7 RNAP activity assay, the reaction mixture contained 50 \(\mu\)g/ml plasmid DNA, 1 \(\mu\)M each of ATP, GTP, CTP and UTP, 0.25 \(\mu\)Ci of \(\alpha\-[\text{32P}]\)-UTP and T7 RNAP (100 nM T7 RNAP in the absence or presence of different concentrations of the dye separately incubated at 20°C for 15 min) in transcription buffer (20 mM Tris-HCl, pH 8.0, containing 1 mM DTT, 10 mM MgCl₂ and 4 mM spermidine). Transcription was allowed to occur at 37°C for 15 min after which the reaction was stopped by freezing to -20°C. The mixture was thawed on ice and spotted on two Whatman GF/C glass fiber filters. One of the filters was allowed to dry, washed with 5% ice-cold solution of TCA, followed by 70% (v/v) ethanol and dried. The filter-retained count, which is the count incorporated into the nascent RNA, was measured by liquid scintillation counting in a LKB 1211 Rack Beta liquid scintillation counter. Total applied count was obtained by measuring the counts of the other unwashed filter. Percentage incorporation was calculated from the ratio of the incorporated count and total count. The residual activity was plotted as a function of input concentration of cibacron blue.

Binding of UTP to cibacron blue-treated T7 RNAP

100 nM T7 RNAP in TDMK buffer, pH 8.0, either free or treated with 1.5 \(\mu\)M or 10 \(\mu\)M cibacron blue, was titrated with \(\alpha\-[\text{32P}]\)-UTP to study the UTP binding capacity of the enzyme in the presence of the dye. Increasing concentrations of labeled UTP (specific activity 0.4 \(\mu\)Ci/n mole) was added separately to 100 nM T7 RNAP taken in TDMK buffer, pH 8.0. After incubation of the mixture at room temperature for 15 min, the mixture was spotted to GF/C filters and allowed to dry. They were then washed with TDMK buffer, dried, and the filter-retained count was measured by liquid scintillation counting. Non-specific filter-retained count was measured by spotting the same amounts of labeled UTP in the absence of polymerase. For each input concentration of UTP, this non-specific count was subtracted from the polymerase bound count and the
difference was plotted as a function of UTP concentration.

Results

Difference absorption spectroscopy

Difference absorption spectra of cibacron blue in the presence of T7 RNAP as shown in Fig. 1 (panels a and b) provides evidence for the association of the dye with the polymerase. A bi-phasic binding is indicated by the nature of the representative spectra and absence of a single isosbestic point over the total range of input concentrations of cibacron blue. At lower concentrations of the dye, up to 1.6 μM, there is a single positive peak at 686 nm. At dye concentrations above 1.6 μM, the nature of the difference spectra changes and double minima appear at 595 nm and 630 nm, respectively. A plot of the peak height at 686 nm as a function of input concentration of the dye (Fig. 2) substantiates the bi-phasic character. A high affinity binding ($K_d = 600$ nM, evaluated from the midpoint of the titration curve) is followed by a second phase ($K_d = 2.5$ μM) in

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Fig. 1—Representative difference absorption spectra of cibacron blue bound to T7 RNAP (1.5 μM) in TDMK buffer, pH 8.0, at 25°C: [The two spectra are representative of the two phases]

![Graph showing representative difference absorption spectra of cibacron blue bound to T7 RNAP (1.5 μM) in TDMK buffer, pH 8.0, at 25°C.]

Fig. 2—Spectrophotometric titration of T7 RNAP (1.5 μM) with cibacron blue in TDMK buffer, pH 8.0 at 25°C. [The absorbance at 686 nm after appropriate subtraction of contribution from buffer containing the enzyme, is plotted as a function of input concentration of cibacron blue. The two panels show the two phases of binding]

![Graph showing spectrophotometric titration of T7 RNAP (1.5 μM) with cibacron blue in TDMK buffer, pH 8.0 at 25°C. The absorbance at 686 nm is plotted as a function of input concentration of cibacron blue, showing two phases of binding.]

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the binding isotherm. The difference spectra corresponding to the second phase are similar to those reported for other RNA polymerases like yeast RNA polymerase I (ref. 23) and Azotobacter vinelandii RNA polymerase24, but the spectra corresponding to the first phase are quite different. The stoichiometry of binding was found to be one dye molecule per polymerase molecule in the first phase and two dye molecules per enzyme molecule in the second phase.

**Fluorescence spectroscopy**

We have also employed fluorescence spectroscopy as an alternative method to monitor quantitatively the high affinity binding. This has been possible because of the high quantum yield of T7 RNAP which contains 18 tryptophan residues. Fluorescence emission intensity of the enzyme at 340 nm is quenched upon progressive addition of increasing concentrations of cibacron blue to T7 RNAP. The emission intensity of the polymerase at 340 nm as a function of input concentration of the dye is shown in Fig. 3. A bi-phasic binding profile was obtained, further indicating that the dye has two binding sites in the polymerase. Half maximal change in fluorescence intensity in the first phase occurs at 500 nM dye concentration, comparable to that obtained from difference absorption studies.

**Inhibition of enzyme activity**

Having observed that T7 RNAP binds to cibacron blue, the effect of this binding on the enzymatic activity of the protein was checked. The percentage incorporation of UMP into RNA by T7 RNAP after treatment with different concentrations of the dye is plotted in Fig. 4. This figure shows that the transcriptional activity of the enzyme is inhibited by cibacron blue and there is a substantial reduction in the activity even upon treatment with 1.0 μM dye. Half maximal inhibition by the dye corresponds to a dye concentration of 200 nM, in the same range as K_d corresponding to the first phase of binding determined from absorption and fluorescence spectroscopy.
Fig. 5—of[^32P]-UTP binding to free and cibacron blue bound T7 RNAP in TDMK buffer, pH 8.0 at 20°C. (Free T7 RNAP, (O); T7 RNAP treated with 1.5 μM cibacron blue, (□); T7 RNAP treated with 10 μM cibacron blue, (△))

Binding of UTP to cibacron blue-bound T7 RNA polymerase

The nucleotide binding site in T7 RNA polymerase is common for all, ATP, GTP, CTP and UTP. So, we have monitored the effect of cibacron blue binding on nucleotide binding at this site by studying the direct binding of radiolabelled UTP to the free and cibacron blue-treated T7 RNAP. The results are shown in Fig. 5. UTP binding is not significantly affected in case of 1.5 μM cibacron blue-treated T7 RNAP. In contrast, pretreatment of the enzyme with 10 μM dye abolishes its UTP binding capacity. These results imply that the second phase of dye binding may correspond to binding at the common nucleotide substrate binding site.

Discussion

Cibacron blue is well known to bind to proteins that utilize nucleotide substrates and cofactors. Since nucleoside triphosphates are substrates of T7 RNA polymerase, it was checked whether there are any binding sites for cibacron blue in T7 RNAP. In this paper, the results of the interaction of T7 RNA polymerase with cibacron blue are presented and this is the first report of such an interaction of the dye with a viral RNA polymerase. Previously, there were reports of binding of a bacterial (Azotobacter vinelandii) DNA polymerase[^23] and an eukaryotic (yeast) RNA polymerase I (ref. 24) with cibacron blue. We have observed that T7 RNAP can bind to cibacron blue in a bi-phasic manner. Such bi-phasic binding has also not been shown for any other RNA polymerase.

Absorption spectroscopic studies show that the high affinity site has a dissociation constant in the nanomolar order with a binding stoichiometry of one dye molecule per molecule of the protein, suggesting that the binding is specific. The high affinity binding could also be detected from the cibacron blue induced quenching of the intrinsic fluorescence of the enzyme. Effect of the high affinity binding upon the spectroscopic and biochemical properties of the enzyme is shown in Fig. 6 where changes in difference absorption in the first phase, fluorescence emission at 340 nm and transcriptional activity are plotted together. They can be fitted to a single curve indicating that all of them originate from the high affinity binding between the dye and the protein. Thus, binding of the dye at a high affinity site causes abolition of the transcriptional activity of the polymerase.

Saturation of the high affinity site with 1.5 μM cibacron blue does not abolish the substrate binding, while saturation of both nucleotide binding sites of the enzyme by 10 μM cibacron blue leads to a loss of substrate binding capacity of the polymerase. The high affinity binding at the first phase itself is...
accompanied by loss in transcriptional activity of the enzyme. We, therefore, conclude that high affinity binding occurs at a site other than the substrate binding site and this site is essential for the transcriptional activity of the enzyme. Further studies are underway to characterize the site and check if there is any correlation between this site and the high affinity GTP binding site reported earlier from our laboratory. 

References